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The Seroepidemiology of Genogroup 1 and Genogroup 2 Norwalk-Like Viruses in Italy

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Southampton virus (SV) and Lordsdale viruses (LV) are small round structured viruses characterised recently and belong to two separate genogroups. The capsid genes of these viruses were expressed in insect cells using recombinant baculoviruses. Both SV (genogroup 1) and LV (genogroup 2) capsid proteins self-assembled to form virus-like particles (VLPs). The VLPs were used in a standard enzyme-linked immunosorbent assay (ELISA) to screen for antibodies to SV and LV in 1,729 age-stratified human sera collected in Verona, Italy between January and November 1996. SV VLPs were labile compared with LV VLPs. There was a large difference in the prevalence of SV (28.7%) compared with LV (91.2%). However, presentation of SV VLPs using chicken egg yolk antibody-coated wells (IgY capture ELISA) with a subset of serum samples from patients (0–19 years) increased the number of positive sera significantly (50.5%), indicating that SV antigen integrity is an important factor in the assay. Recent reverse transcription-polymerase chain reaction (RT-PCR) studies have shown that LV is circulating currently and analysis of IgY capture ELISA data showed greater reactivity for LV than SV, reflecting a genuinely lower rate of recent infection by this genogroup 1 virus. ***J. Med. Virol. 58:93–99, 1999.*** © 1999 Wiley-Liss, Inc.

KEY WORDS: Southampton virus; Lordsdale virus; Norwalk virus; ELISA; serology

INTRODUCTION

Small round structured viruses (SRSVs) or Norwalk-like viruses (NLVs) are recognised worldwide as the most important cause of epidemic nonbacterial gastroenteritis in adults. Rotaviruses, however, are the main cause of outbreaks of acute gastroenteritis in children. Volunteer studies have increased our knowledge and understanding of the transmission and immunobiology of the NLVs [Clarke et al., 1997]. Characteristically, NLVs cause acute diarrhoea and vomiting with rapid secondary spread arising from person-to-person transmission [Caul, 1996]. Outbreaks involving large numbers of people have been described that usually occur in settings where people congregate, for example, cruise ships, hospitals, schools, hotels, and nursing homes. Large point-source outbreaks of NLV infections have been associated with contamination of water and food [SteneJohansen and Grinde, 1996].

The virions measure 32–38 nm in diameter and have a positive polarity single-stranded RNA genome of approximately 7.5 kb [Clarke and Lambden, 1997]. Complete genome sequences of three NLVs have been characterised representing the UK Southampton virus [Lambden et al., 1993, 1995] and Lordsdale virus and the prototype Norwalk virus from the USA . This work has allowed the definitive classification of these NLVs within the virus family *Caliciviridae.* Comparative sequence analysis of selected regions of the genome from a wider number of NLVs has shown that these viruses can be divided into two distinct genetic groups [Lambden and Clarke, 1995]. The viruses from the two genetic groups share a similar overall genome structure, although capsid genes show significant sequence diversity [Lambden and Clarke, 1995]. Norwalk virus (NV) and Southampton virus (SV) belong to genetic group 1, whereas Lordsdale virus (LV) and a number of other partially characterised strains, Toronto virus (TV) [Lew et al., 1994b], Mexico virus (MX) [Jiang et al.,

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1995], and Hawaii virus (HV) [Lew et al., 1994a] are members of genetic group 2.

The lack of a cell culture system for NLVs has impeded the production of reagents for laboratory studies. However, the difficulties of obtaining sufficient virus antigen to study the immunobiology and seroepidemiology of NLVs have been overcome by the expression of the capsid protein in insect cells using recombinant baculovirus vectors. This production was first achieved for Norwalk virus [Jiang et al., 1992] and has been accomplished subsequently for MX [Jiang et al., 1995], TV [Leite et al., 1996], HV [Green et al., 1997], and LV [Dingle et al., 1995]. For each of these different NLVs, the expressed capsid proteins undergo self-assembly into virus-like particles (VLPs).

Recombinant Norwalk virus (r-NV) antigen performs similarly to natural NV antigen in a range of assays [Green et al., 1993]. Extensive seroepidemiological surveys have been undertaken using r-NV antigen [Gray et al., 1993; Green et al., 1993; Monroe et al., 1993; Taylor et al., 1993; Treanor et al., 1993; Gabbay et al., 1994; Graham et al., 1994; Lew et al., 1994c; Numata et al., 1994; Parker et al., 1993, 1994] and more recently r-MX [Jiang et al., 1995; Dimitrov et al., 1997]. These studies suggest a high seroprevalence of both NV and MX in the general community.

The purpose of this study was to express recombinant capsid proteins from European genogroup 1 and genogroup 2 NLVs (SV and LV) for use in a solid phase antibody capture assay. Two separate immunoassays for SV and LV were developed and applied to a large number of age-stratified human serum samples to compare the seroprevalence of these strains representing the two NLV genogroups.

MATERIALS AND METHODS

Construction of Recombinant Baculovirus Transfer Vectors

Construction of a recombinant baculovirus carrying the open reading frames (ORFs) 2 and 3 and the 3′ UTR of Lordsdale virus has been described previously [Dingle et al., 1995]. For SV, a 2.35-kb polymerase chain reaction (PCR) fragment containing ORF2, ORF3, and the 3′ UTR was generated using a full-length cloned copy of the SV genome (pT7SVpolyA) as template with primer pair SVSUB (5′-^{5351}GTAAATGATGATGGCGTCTA^{5370}-3′) and SV37 (5′-TCGAGATCT^{7708}AACACTAATCAATAGCCAAATT^{7687}-3′). The underlined sequence in primer SV37 indicates a *Bgl* II site synthesised on the authentic SV primer sequence to facilitate subsequent cloning into the baculovirus transfer vector. Prior to cloning into the pBlueBac II baculovirus transfer vector, the 2.35-kb PCR fragment was ligated into *Sma* I-cleaved pSP64polyA (Promega, Southampton, UK) for sequence verification. The insert was released by digestion of the vector with *Bam* HI and *Bgl* II and the purified fragment was ligated into *Bam* HI-digested dephosphorylated pBlueBac II vector. Transformants were screened by PCR using primers PBBIIF (5′-^{4682}ACGTTGAACTCGCCGCAG^{4665}-3′) and SV21 (5′-^{6275}TGGTTTGCCATCCACCTC^{6257}-3′); recombinants carrying inserts in the correct orientation were chosen for transfection into insect cells.

Insect Cell Culture

Monolayers of Sf9 (*Spodoptera frugiperda*) insect cells at approximately 80% confluence were cotransfected with linear wild type baculovirus DNA (*Autographa californica* nuclear polyhedrosis virus AcNPV) and recombinant transfer vector using cationic liposomes (Invitrogen, Carlsbad, CA). Recombinant viruses were identified initially by PCR and were then purified by three rounds of single plaque isolation. High titre stocks of recombinant baculoviruses were produced in Sf9 cells grown in TC100 medium and stored as described previously [King and Possee, 1997].

Expression of r-SV and r-LV Capsids and Purification of VLPs

Expression studies were performed using High-Five (HF) cells from the cabbage looper (*Trichoplusia ni*) grown in a low protein, serum-free medium (EX-CELL 400, JRH Biosciences, Lenexa, KS). Following adsorption of recombinant baculoviruses to the cells at a multiplicity of infection of 10, cell monolayers were washed with phosphate-buffered saline (PBS) to remove the inoculum, and viral protein expression was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Time course experiments were carried out over a 5-day period to determine the optimum time to harvest capsid proteins. Electron microscopic (EM) examination of the cell culture supernatant showed that viral capsid protein undergoes self-assembly to form VLPs for both SV [Clarke et al., 1997] and LV [Dingle et al., 1995]. VLPs were purified on CsCl gradients following removal of cellular debris by low-speed centrifugation of the cell culture supernatant. Crude VLPs were pelleted by centrifugation for 2 hr at 28,000 rpm using a Beckman SW28 rotor, resuspended in CsCl at 1.362 g/cm^3, and run for 24 hr at 35,000 rpm to reach equilibrium in a Beckman SW50.1 rotor. CsCl was removed from gradient fractions containing VLPs by dialysis with 10 mM Tris-HCl, 150 mM NaCl pH 7.5. Purified VLPs were stored in aliquots at −135°C.

Production of Antisera

Hyperimmune antisera to purified r-SV and r-LV VLPs were produced in laying hens. A primary immunisation of purified VLP protein (100 μg) was administered intramuscularly (IM) in complete Freund's adjuvant followed by three boosts IM in incomplete Freund's adjuvant at 10-day intervals. Chicken immunoglobulin from egg yolks (IgY) at 57 days post-immunisation was purified using the EGGstract IgY Purification System (Promega, Southampton, UK). These hyperimmune sera were used to optimise conditions for the enzyme-linked immunosorbent assay (ELISA) to detect serum antibodies to SV and LV.

ELISAs

To detect LV- and SV-specific IgG, two separate ELISAs were carried out using 96-well polyvinylchloride microtiter plates (ICN-FLOW, Oxfordshire, England). For the direct ELISA, plates were coated at 4°C overnight with r-LV or r-SV capsid antigen in 0.05 M carbonate-bicarbonate buffer (pH 9.6) in a volume of 100 μl/well. Optimisation of antigen coating established that well saturation was achieved with 100 ng/well of r-LV and with 200 ng/well of r-SV. After overnight incubation, wells were blocked with 200 μl of 5% skim milk powder (Carnation nonfat milk) in PBS, pH 7.2, for 30 min at 37°C and then washed twice with a solution of 0.05% v/v Tween 20 in PBS. Patients' serum samples were added at a dilution of 1:100 in 1% skim milk powder in PBS at a volume of 100 μl/well. The serum dilution of 1/100 was the optimum working dilution for human sera. Each plate also contained positive and negative serum controls together with conjugate and substrate controls. Positive control reference sera were obtained from patients following natural infection with either SV or LV. Pools of negative sera were considered suitable for use as controls if they exhibited a low reactivity that was not reduced further by dilution. After a 60-minute incubation at 37°C and three washes as described above, 100 μl of goat anti-human IgG peroxidase-conjugate (γ-chain specific, Sigma Chemical Company, St. Louis, MO) diluted 1:5,000 in 1% skim milk powder in PBS, was added to each well except for the substrate control wells. The plates were incubated at 37°C for 1 hr and washed four times as described previously. Then, 100 μl of a freshly prepared tetramethylbenzidine solution (Sigma) at a concentration of 0.036 mg/ml in 0.01 M sodium acetate buffer (pH adjusted to 6 with citric acid) containing H_2O_2 (30%) diluted 1:10,000 was added to each well. After 12 min incubation at room temperature, the reaction was stopped by adding 50 μl of 2 M sulphuric acid to each well. The optical density was read at a wavelength of 450 nm in a microtitre plate reader (Anthos htII, Salzburg, Austria). Sera were considered antigen reactive, and therefore antibody positive, when the ratio of their optical density over the mean optical density of the negative control was 3 or greater. The interassay variation of the mean optical density of the negative control ranged between 0.065 and 0.085. Serum prevalence of anti-LV and anti-SV antibodies was calculated by age group in the range 1 day (newborn) to 95 years.

Sandwich ELISAs were conducted as follows: plates were coated with either LV- or SV-specific IgY diluted 1/4,000 (optimum dilution) in 0.05 M carbonate-bicarbonate buffer (pH 9.6) in a volume of 100 μl/well overnight then blocked with 200 μl 5% skim milk powder in PBS for 30 min at 37°C and washed twice with 0.05% Tween 20 in PBS. Recombinant SV and LV were added (100 μl/well) at concentrations of 1 μg/ml and 2 μg/ml, respectively. Following incubation at 37°C for 1 hr, the plates were washed four times with 0.05% Tween 20 in PBS. A subset of the serum samples (n = 525; age range, newborn to 19 years) were then added at 1/100 dilution and assayed for SV and LV IgG as in the standard procedure described above.

Patients' Sera

Serum samples were collected randomly in Verona, north-eastern Italy, from 1,729 patients between January and November 1996. The sera were stored at –80°C prior to use. The sera were from both hospital patients and patients attending outpatient clinics at the "Azienda Ospedaliera di Verona." The age range of these patients was between 1 day and 95 years; 53.8% of the patients were female, 46.2% were male. There was no association between collection of the samples and presence or absence of known recent gastrointestinal disease.

Statistical Methods

The Fisher exact t test was used to detect statistically significant variations in seroprevalence between age groups.

RESULTS

Expression of SV and LV Capsids

Expression of r-SV and r-LV capsids was followed over a 5-day period. For SV, capsid protein was detectable in infected cells on the first day after infection. From the second day postinfection, capsid protein synthesis in the cells was maximal together with an additional product of 34 kDa. The 34-kDa protein was observed as a major band in infected cells and it remained cell-associated, whereas the full-length 58-kDa capsid protein was released to the cell culture supernatant on days 3–5, reaching a maximum on day 5 (Fig. 1A). The expression kinetics for r-LV were similar, although two major protein bands of 57 and 52 kDa appeared in the cell culture supernatant and only a minor band of 34 kDa was observed in the infected cells (Fig. 1B). Following equilibrium density gradient centrifugation, both SV and LV VLPs gave a single visible band with a buoyant density of 1.36 g/cm^3. SDS-PAGE analysis of purified VLPs showed r-SV was comprised solely of the 58-kDa product, whereas the equivalent fraction of r-LV contained both the bands (57 and 52 kDa) observed previously in the cell culture supernatant. Direct EM examination of freshly prepared VLPs in 10 mM Tris-HCl pH 7.5 showed good particle structure for both recombinants, although storage in this buffer at 4°C for periods longer than 1 week showed significant particle degradation for r-SV. In contrast, r-LV VLP structure was well preserved for up to 1 month at 4°C. To maintain particle integrity, aliquots of freshly prepared VLPs from both recombinants were stored at –135°C and thawed immediately prior to use.

Direct ELISA to Detect LV and SV Antibodies

Sera from 1,729 patients with an age range of 1 day to 95 years were tested in a standard ELISA format in which r-LV and r-SV VLPs were used to coat a 96-well

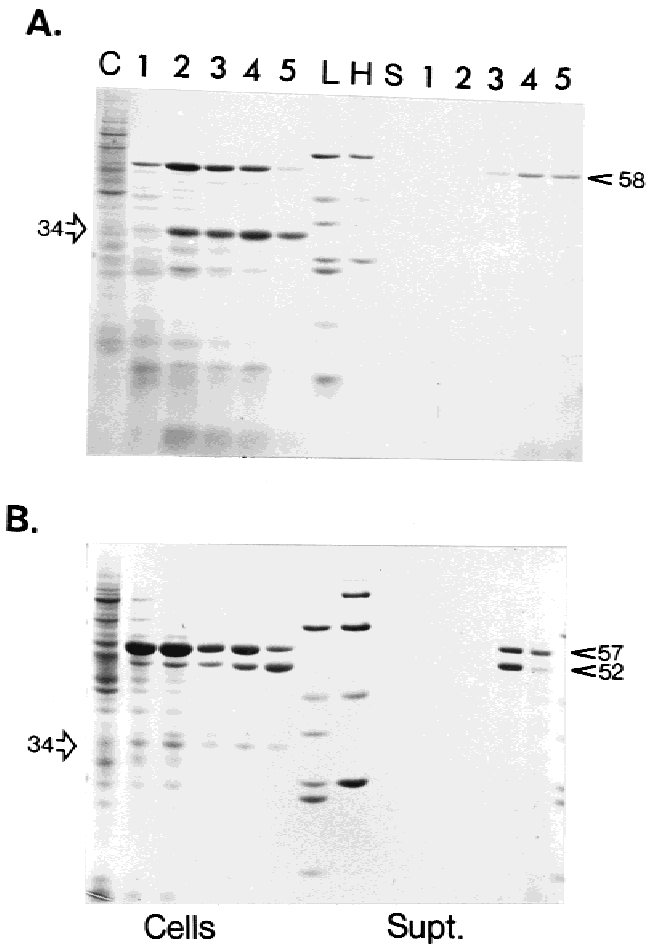

Fig. 1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis showing the time course of recombinant antigen production over a 5-day period from **(A)** Southampton virus (SV) and **(B)** Lordsdale virus (LV). The tracks to the left of the molecular weight markers (1–5) show antigen associated with High Five cells and tracks to the right of the molecular weight markers (1–5) show antigen accumulation in the cell culture supernatant. Mock-infected cells and supernatant are shown in tracks C and S, respectively. Track L contains low molecular weight markers and track H contains high molecular weight markers. The open arrow on the left of both panels shows the cell-associated capsid proteolysis product of 34 kDa. The unprocessed SV capsid is indicated at 58 kDa. In LV, two products of 57 and 52 kDa are associated with cell-free recombinant antigen. The proteins were visualised by staining with Coomassie blue.

microtitre plate. The prevalence of LV antibody by age group is shown in Table I.

The majority of sera from children younger than 6 months (73.7 %) were reactive. However, for children between 6 and 11 months of age, the percentage of reactive sera fell to a value of 44.4% ($P = .017$) and then increased sharply during the second and third years of life when 70.9% of the sera were positive ($P = .0052$). During the fourth and fifth year of life the rise in the percentage of reactive sera was less pronounced, reaching the value of 87.8% ($P = .0036$). By the ages of 5–9 years, the percentage of positively reacting sera increased further to 94.8%, reaching a peak value of 96.3% in the fourth decade. Overall 1,576 of 1,729 (91.2%) serum samples were positive for LV antibody.

Table I also shows the prevalence of SV antibodies in the same population. Of the sera collected from children under 6 months of age, 13.2% were reactive for SV; between 6 and 11 months the percentage of positive sera decreased to a value of 8.3% then rose to 26.4% between 12 and 35 months of age. In the age group 36–59 months, reactivity reached 36.7% and then peaked at 5–9 years (38.5%). Thereafter, the seroprevalence declined and remained below 30% in the fourth decade and beyond. In sharp contrast to LV, only 497 (28.7%) serum samples were positive for SV.

Sandwich ELISA for the Detection of LV- and SV-Specific Antibodies

The unexpected large difference in antibody prevalence for LV and SV in the direct assay led us to investigate whether assay presentation of recombinant antigen affects the reactivity of human sera. For this analysis, 96-well trays were coated with chicken IgY and used to bind r-LV and r-SV antigen purified as VLPs. The seroprevalence study was repeated using the original sera from Verona representing the age range newborn (1 day) to 19 years ($n = 525$). Comparison of the seroprevalence of anti -LV antibodies detected by either method showed no significant differences, 86.5% by the direct antigen assay and 83.8% by the chicken IgY sandwich ELISA. The average OD_{450} of r-LV-positive samples decreased only marginally using the direct coating of antigen compared with chicken antibody capture of r-LV antigen. In contrast, the seroprevalence for SV increased (by 38%) when r-SV antigen captured by chicken antibody was used (Fig. 2) and the total number of sera reactive with r-SV increased from 163 (31%) to 265 (50.5%). The distribution of OD_{450} values for the sera collected in Verona (age range, newborn to 19 years) for SV and LV using specific IgY-captured antigen are shown in Figure 3.

DISCUSSION

The low levels of antigen shed during NLV infection and the lack of a cell culture system for these viruses has hampered the development of specific reagents for the detailed study of their seroepidemiology. Thus native SV and LV antigens have not been tested for cross reactivity with other NLVs; however, phylogenetic studies suggest that these viruses are genetically distinct from the prototype strains NV, HV, and TV/MX [Noel et al., 1997]. The use of antigens produced by recombinant baculoviruses in insect cells has permitted preliminary studies of the seroepidemiology of the prototype NLVs.

In our study, SV and LV VLPs were produced using HF cells because this cell line grows without serum and gives significantly higher expression of the same proteins than Sf9 cells grown in serum-free medium. In HF cells the time course expression patterns for both SV and LV were similar. Of the two major SV proteins expressed (34 and 58 kDa), only the polypeptide of 58 kDa was released into the cell culture supernatant and formed VLPs. This observation is consistent with bacu-

TABLE I. Prevalence of Antibodies to Lordsdale Virus (LV) and Southampton Virus (SV) in Verona, Italy

Age range	Number of samples	Positive samples (LV)		Positive samples (SV)	
		No.	%	No.	%
<6 months	38	28	73.7	5	13.2
6–11 months	36	16	44.4	3	8.3
1–2 years	110	78	70.9	29	26.4
3–4 years	98	86	87.8	36	36.7
5–9 years	96	91	94.8	37	38.5
10–19 years	147	141	95.9	53	36.1
20–29 years	239	224	94.9	76	32.2
30–39 years	187	180	96.3	46	24.6
40–49 years	158	151	95.6	37	23.4
50–59 years	200	186	93.0	53	26.5
60–69 years	213	199	93.4	60	28.2
>70 years	210	196	93.3	62	29.5
Total	1729	1576	91.2	497	28.7

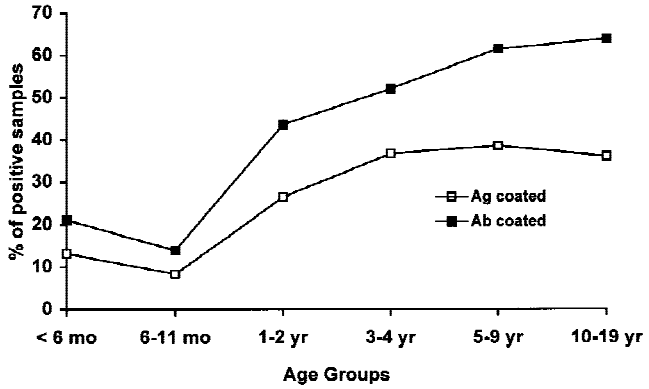

Fig. 2. Detection by enzyme immunoassay of anti-Southampton virus IgG form the patient's sera collected in Verona, Italy: Comparison using two separate antigen presentation formats (antigen vs. antibody/antigen complex-coated wells).

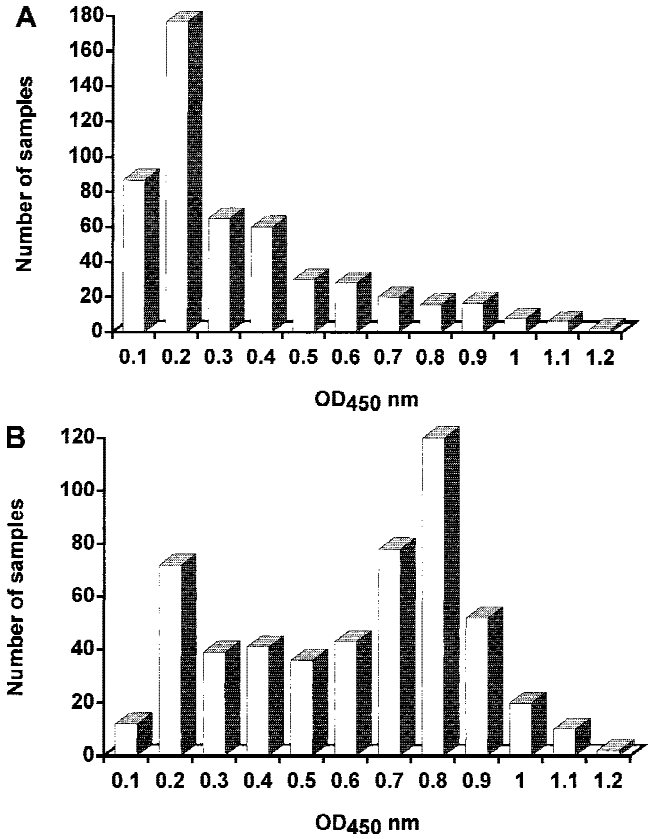

Fig. 3. Distribution of OD_{450} values of the sera collected in Verona, Italy (age range, newborn to 19 years) for **(A)** Southampton virus (SV) and **(B)** Lordsdale virus (LV) using specific IgY-captured antigen.

lovirus-expressed NV, in which a 34-kDa protein was also found only in association with infected insect cells [Jiang et al., 1992]. Amino terminal sequence analysis of a 32-kDa product produced following disassembly of r-NV VLPs showed that that this protein was a specific proteolytic cleavage product of r-NV capsid [Hardy et al., 1995]. Expression of r-LV results in a small amount of 34-kDa product, however two major proteins of 57 and 52 kDa are exported to the cell culture supernatant and recovered with the gradient-purified VLPs. It is not known if the 52-kDa band is a specific proteolytic cleavage product of the 57-kDa protein or a separate translation product resulting from aberrant internal initiation.

Previous studies with r-MX VLPs showed that prolonged storage at 4°C resulted in the degradation of VLP structure and the appearance of small round (15–20 nm) particles [Jiang et al., 1995]. Such small round particles have also been observed in purified preparations of r-HV and r-NV [Green et al., 1997; White et al., 1997]. In the case of r-NV, the small particles are not composed of degraded capsid protein but seem to be a structural rearrangement of the full-length capsid protein. Thus to maintain the quality and preserve the antigenic structure of the r-SV and r-LV VLPs, gradient-purified particles were dialysed against 10 mM Tris-HCl (pH 7.5) and stored at –135°C.

Studies using recombinant VLPs for NV and MX capsid proteins showed high levels of antibody in the general population [Gray et al., 1993; Gabbay et al., 1994; Lew et al., 1994c; Numata et al., 1994; Parker et al., 1994; Dimitrov et al., 1997]. These findings were not unexpected because NLVs are considered to be highly

infectious and often circulate in the community. In addition, volunteer studies have shown there is no long-term antibody-mediated protection against reinfection by the same NLV [Blacklow et al., 1972; Johnson et al., 1990; Gray et al., 1994], suggesting that individuals may encounter the same virus several times during a lifetime.

Our survey was designed to investigate the distribution of antibodies in the same population for two different genogroups of NLVs using sera collected from patients in Verona, Italy. Based on previous studies with NV and MX, we expected to see an initial high level of serum antibodies in the < 6 months age group (representing maternal antibodies) followed by a progressive decay and then rising levels as children acquire antibodies to LV and SV from exposure to the viruses. However, the direct ELISA on human sera demonstrated a significant difference in the overall seroprevalence for LV (91.2 %) compared with SV (28.7%). This observation was surprising considering that previous assays for r-NV and r-MX virus had shown generally high seroprevalence for both genetic group 1 and 2 viruses in other populations [Gray et al., 1993; Gabbay et al., 1994; Lew et al., 1994c; Numata et al., 1994; Parker et al., 1994; Dimitrov et al., 1997]. In these studies, there were only small differences in the general age prevalence of MX and NV antibodies that may be accounted for by differences in assay protocols and conditions, choice of cut-off values, and geographic areas from where the populations were surveyed. However, none of the differences were as large as those observed between the seroprevalence values of SV and LV.

Explanations for these major differences in seroprevalence between SV and LV may include that the r-SV antigen loses common reactive epitopes during coating of the 96-well microtitre trays or that the low seroprevalence reflects a genuinely lower infectivity of the SV, which is now known to be not as common as LV in the community [Green et al., 1997]. The relatively poor stability of r-SV VLPs compared with r-LV VLPs plus the recent observation that r-NV particles are unstable at pH 8.5 [Hardy et al., 1995] led us to investigate whether antigen presentation affected serum reactivity. Some denaturation must occur when VLPs are coupled directly to solid-phase surfaces, especially at high pH. Thus to preserve the antigenic integrity of the r-SV, specific antibody was used to coat the wells so that antigen could be captured and presented in a native state without exposure to high pH coating buffer or binding directly to the solid phase. Chicken IgY was chosen to coat wells because of the low levels of cross-reaction between chicken IgY and the secondary antibodies [Ambrosius and Hadge, 1987] used in the assay. Data obtained from the sandwich ELISA showed a significant increase (50.5%) in the seroprevalence of SV (Fig. 2) and no change for LV (data not shown). Thus in the case of SV, preservation and presentation of the antigen in the assay has a significant effect on the observed reactivity of the human sera, giving similar seroprevalence rates to NV as described in the most recent seroepidemiological survey of NV in the UK [Cubitt et al., 1998]. A recent study investigating the correlation of immune responses with infecting viruses [Noel et al., 1997] has shown that within genogroup 2 viruses the specificity of human serum responses varied widely. However, patients infected with genogroup 1 viruses (with divergent capsid sequences) demonstrated homogeneous responses with r-NV VLPs. Thus our hypothesis is that human antibodies to genogroup 1 viruses react broadly and antibody responses to genogroup 2 viruses are generally more specific. The lower seroprevalence of genogroup 1 viruses presented here is supported by the reduced detection of genogroup 1 viruses from 1990 to 1995 [Noel et al., 1997].

In the UK, LV remains a common cause of nonbacterial gastroenteritis [Green et al., 1997], but we have not detected SV in clinical specimens from patients during the period of this study. Interestingly, we and other independent workers [Parker et al., 1993] have never detected NV in faecal specimens from any outbreaks of gastroenteritis in the UK. Thus the lower seroprevalence of SV and NV appears to reflect a genuinely lower rate of infection by the genogroup 1 viruses in the population. This conclusion is further supported by our finding that although some sera react strongly for SV (Fig. 3A), most of the positive sera have lower absorbency (average OD_{450} amongst these positive samples is 0.49) than LV. In contrast, for LV there is a "U-shaped" distribution of absorbance values (Fig. 3B) and many more sera have high OD_{450} (average OD_{450} amongst the positive samples was 0.64), suggesting recent infection and thus supporting the observation that LV-like viruses continue to circulate [Green et al., 1997].

In conclusion, some of the observed differences in seroprevalence between SV and LV are due to the relatively poor stability of the r-SV in the standard ELISA. However, the difference in positivity rates with the IgY antigen capture ELISA probably reflects a genuine lower incidence of recent infection with SV in the population tested. Moreover, detailed understanding of the epidemiology of these viruses must also consider the genetic variation of circulating strains in conjunction with seroepidemiological studies. Until a standardised panel of diagnostic reagents become available, we have to consider the possibility that NLVs from the two genetic groups have varying pathogenic properties and display variable infectivity patterns amongst different age groups within communities.

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